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# Gamma Interferon, Tumor Necrosis Factor Alpha, and Nitric Oxide Synthase 2, Key Elements of Cellular Immunity, Perform Critical Protective Functions during Humoral Defense against Lethal Pulmonary *Yersinia pestis* Infection

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**Pulmonary infection by *Yersinia pestis* causes pneumonic plague, a rapidly progressing and often fatal disease. To aid the development of safe and effective pneumonic plague vaccines, we are deciphering mechanisms used by the immune system to protect against lethal pulmonary *Y. pestis* infection. In murine pneumonic plague models, passive transfer of convalescent-phase sera confers protection, as does active vaccination with live *Y. pestis*. Here, we demonstrate that protection by either protocol relies upon both gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) cytokines classically associated with type 1 cellular immunity. In both protocols, abrogating IFN-γ or TNF-α activity significantly decreases survival and increases the bacterial burden in pulmonary, splenic, and hepatic tissues. Neutralization of either cytokine also counteracts challenge-induced, vaccination-dependent upregulation of nitric oxide synthase 2 (NOS2). Moreover, genetic depletion of NOS2 suppresses protection conferred by serotherapy. We conclude that IFN-γ, TNF-α, and NOS2, key elements of cellular immunity, perform critical protective functions during humoral defense against lethal pulmonary *Y. pestis* challenge. These observations strongly suggest that plague vaccines should strive to maximally prime both cellular and humoral immunity.**

Pneumonic plague is one of the most feared infectious diseases in recorded history. The etiologic agent of plague is *Yersinia pestis*, a facultative intracellular gram-negative bacillus that causes several different forms of disease (30). The least deadly form, bubonic plague, results from the bite of an infected flea and is characterized by painfully swollen lymph nodes, also known as buboes. When left untreated, bubonic plague can progress to the more deadly septicemic and pneumonic forms. The latter is highly contagious, rapidly progressing, and associated with high mortality.

There is no safe and effective pneumonic plague vaccine. Killed whole-cell vaccines protect against bubonic, but not pneumonic, plague (26, 39). Live attenuated *Y. pestis* vaccines protect mice and guinea pigs against pneumonic challenge, and limited data indicate that they also protect humans, but reactogenicity and safety concerns preclude their widespread use (16, 26, 39). It is widely acknowledged that military scientists formulated methods to aerosolize virulent *Y. pestis* organisms. If diagnosed early, plague can be treated with antibiotics. However, multidrug-resistant *Y. pestis* isolates have been identified (9), thus raising grave concern that antibiotic-resistant *Y. pestis* strains may be used as bioweapons (14).

It has long been recognized that serum samples isolated from plague convalescents can passively transfer protection to naïve mice (25). Given this demonstrated efficacy of humoral immunity, significant effort has been devoted to the development of subunit plague vaccines that generate protective antibody responses. Thus far, the *Y. pestis* fraction 1 (F1) and V proteins appear to offer the most promise. Vaccination with a recombinant F1-V fusion protein protects mice against lethal pulmonary challenge (1, 11, 12), and sera from mice vaccinated with F1 and V confer passive protection (10). Recently, the U.S. Army evaluated the F1-V fusion protein vaccine in nonhuman primates and found that it protected cynomolgus macaques but largely failed to protect African green monkeys (32). The variable efficacy of this vaccine in primates raises concern that humoral immunity directed at F1 and V may not suffice in protecting humans against pneumonic plague.

T-cell-dependent cellular immunity comprises another means by which vaccines can prime long-lived protection against facultative intracellular bacterial pathogens. Cell-mediated protection against intracellular bacteria often relies upon the development of type 1 immune responses, characterized by the expansion of pathogen-specific T cells that secrete gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) (19). These cytokines are pleiotropic and likely combat intracellular bacteria by many mechanisms (23, 35). For example, IFN-γ and TNF-α stimulate macrophages to upregulate expression of nitric oxide synthase 2 (NOS2), thereby increasing their capacity to produce antimicrobial nitric oxide (24).

*Y. pestis* organisms can replicate within the macrophage phagolysosome and are found intracellularly during plague (6, 15, 22, 33, 37). Type 1 cellular immunity could potentially upregulate macrophage microbicidal activities, thereby helping to eradicate these intracellular *Y. pestis* reservoirs. Indeed, administration of IFN-γ and TNF-α prior to challenge protects naïve mice against a lethal systemic *Y. pestis* challenge (28). Moreover, STAT4-deficient mice are unable to generate ro-

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bust type 1 responses (18, 38), and vaccinating with F1 and V fails to protect STAT4-deficient mice against subcutaneous *Y. pestis* challenge, despite eliciting high-titer antibody responses (8). While these observations suggest that type 1 cytokines may play critical roles during vaccine-mediated protection against *Y. pestis* infection, their relevance in the setting of pulmonary *Y. pestis* challenge remains to be determined, as do the underlying protective mechanisms.

Recently, we developed a model that enabled us to specifically investigate protective roles for cellular immunity during pulmonary *Y. pestis* infection. We found that actively vaccinating B-cell-deficient μMT mice with live *Y. pestis* primes T cells that confer protection against lethal pulmonary challenge (29). Here, we report that neutralization of either IFN-γ or TNF-α abrogates that protection. Moreover, we demonstrate that humoral protection mediated by passive serotherapy also relies upon IFN-γ, TNF-α, and NOS2. These findings reveal a previously unappreciated role for cellular defense mechanisms during humoral protection against lethal pulmonary *Y. pestis* infection.

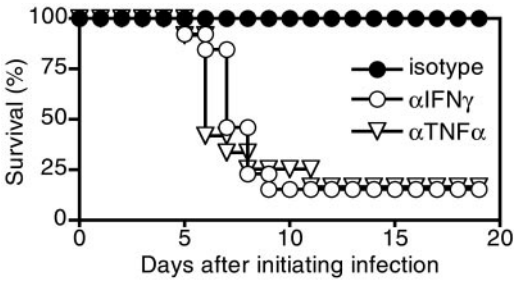


FIG. 1. IFN-γ and TNF-α perform critical protective functions during cell-mediated defense against lethal pulmonary *Y. pestis* challenge. B-cell-deficient μMT mice were actively vaccinated with live *Y. pestis* ($2 \times 10^5$ CFU i.n., followed 18 h later by convalescent-phase sera) and challenged 60 days later ($2 \times 10^5$ *Y. pestis* CFU i.n.). At the time of challenge, mice were treated with neutralizing MAbs specific for IFN-γ (anti-IFN-γ antibody [αIFNγ]) or TNF-α (anti-TNF-α antibody [αTNF-α]) or were treated with isotype-matched control. In comparison with control, neutralization of IFN-γ or TNF-α significantly reduced survival ($P < 0.0001$ for both cytokines; $n = 12$ or 13 mice per group). Data are pooled from two independent experiments.

## MATERIALS AND METHODS

**Mice.** Wild-type, IFN-γ receptor (IFN-γR)-deficient, TNF-α-deficient, NOS2-deficient, and B-cell-deficient μMT mice, each on a C57BL/6 background, were purchased from The Jackson Laboratory (Bar Harbor, ME) or were bred at Trudeau Institute. Animals were cared for according to Trudeau Institute Animal Care and Use Committee guidelines.

**Bacteria.** All experiments employed the pigmentation-negative *Y. pestis* strain KIM D27 (20), which was generously provided by Robert Brubaker (Michigan State University). *Y. pestis* organisms were grown in brain heart infusion broth (Sigma) at 26°C, and infectious stocks were stored as single-use aliquots at −70°C after resuspension in the same medium supplemented with 20% glycerol. The median lethal dose of this stock was approximately $1 \times 10^4$ CFU when administered via the intranasal (i.n.) route, as calculated by the method of Reed and Muench (34).

**Convalescent-phase sera and passive serotherapy protocol.** To generate convalescent-phase sera for passive serotherapy, wild-type mice were inoculated intraperitoneally with $3 \times 10^2$ *Y. pestis* CFU, and serum samples were collected 30 days later (29). The serum samples were pooled, aliquoted, and stored at −20°C. For passive serotherapy, mice were challenged intranasally with 30 μl saline containing $2 \times 10^5$ CFU *Y. pestis*, and 20 μl convalescent-phase sera was administered intraperitoneally 18 h later.

**Active vaccination protocol.** B-cell-deficient μMT mice were inoculated intranasally with 30 μl saline containing $2 \times 10^5$ *Y. pestis* CFU and 20 μl convalescent-phase sera was administered 18 h later (29). At 2 weeks postvaccination, animals received chow supplemented with 67 mg of sulfadiazine per g of body weight and 333 mg of trimethoprim (Uniprim diet; Harlan TEKLAD, Madison, WI) per g and were so maintained until day 55 postvaccination, at which time they were returned to antibiotic-free chow. This antibiotic treatment ensured that the immunocompromised μMT mice did not inadvertently become infected with environmental pathogens prior to challenge infection ($2 \times 10^5$ *Y. pestis* CFU i.n.), which was performed at day 60 postvaccination. Control sham-vaccinated mice also received convalescent-phase serum and antibiotic treatment (29). Where indicated, animals were treated with 1 mg neutralizing monoclonal antibody (MAb) specific for murine IFN-γ (clone XMG1.2) or TNF-α (clone XT3.11). The MAbs were administered as two intraperitoneal doses of 500 μg each on the day before and the day of challenge. Control animals received injections of isotype-matched antibody (rat immunoglobulin G1, clone HRPN). In all survival experiments, recumbent animals were considered moribund and euthanized.

**Measurement of bacterial CFU and NOS2 mRNA levels.** At the indicated days postchallenge infection, mice were euthanized by carbon dioxide narcosis. Spleens, livers, and saline-perfused lungs were harvested and plated for CFU determination as previously described (29). The tissue levels of NOS2 mRNA were measured by real-time PCR, normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase mRNA, and reported as changes relative to the levels in uninfected wild-type mice (17, 36).

**Statistics.** Survival data were analyzed by log-rank tests. All other data were analyzed using Student's *t* tests (GraphPad Prism 4.0 software). Unless otherwise noted, CFU and mRNA data are depicted as means and standard deviations.

## RESULTS

**IFN-γ and TNF-α perform critical protective functions during cell-mediated defense against lethal pulmonary *Y. pestis* challenge.** We previously reported that active vaccination with live *Y. pestis* primes T cells that protect B-cell-deficient μMT mice against lethal pulmonary challenge (29). We hypothesized that IFN-γ and TNF-α may contribute to this protective response, since these cytokines are produced by T cells and their exogenous administration protects naïve mice against lethal systemic *Y. pestis* challenge (28). To investigate functional roles of IFN-γ and TNF-α in a pulmonary challenge model, we actively vaccinated μMT mice and administered neutralizing MAbs specific for either IFN-γ or TNF-α at the time of challenge. As shown in Fig. 1, vaccinated mice treated with either IFN-γ- or TNF-α-specific MAbs exhibited significantly reduced survival in comparison with vaccinated animals that received isotype-matched control MAb ($P < 0.0001$). We conclude that both IFN-γ and TNF-α perform critical protective functions during cell-mediated protection against lethal pulmonary challenge.

To investigate how IFN-γ and TNF-α contribute to protection, we examined bacterial growth in the lungs, livers, and spleens of the vaccinated mice. At day 3 postchallenge, we recovered significantly decreased numbers of CFU from the lungs of actively vaccinated mice compared with those of sham-vaccinated controls (Fig. 2A; $P < 0.0001$). Neutralization of IFN-γ or TNF-α abrogated this vaccination-induced suppression of pulmonary bacterial growth (Fig. 2A; $P = 0.02$ and $P < 0.0005$, respectively). We also recovered significantly decreased numbers of CFU from the livers of actively vaccinated mice (Fig. 2B; $P < 0.0001$), and again, neutralization of either IFN-γ or TNF-α abrogated the vaccination-induced suppression of bacterial growth (Fig. 2B; $P = 0.03$ and $P < 0.0001$, respectively). The recovery of CFU from the spleens was sim-

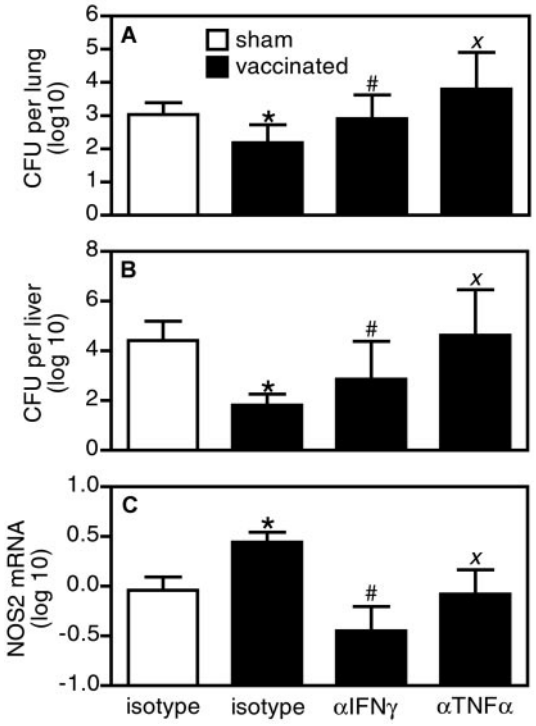


FIG. 2. IFN-γ and TNF-α control bacterial growth and upregulate NOS2 expression during cell-mediated defense against lethal pulmonary *Y. pestis* challenge. B-cell-deficient μMT mice were sham vaccinated or actively vaccinated and challenged 60 days later, as described in the legend to Fig. 1. At the time of challenge, mice were treated with neutralizing MAbs specific for IFN-γ (anti-IFN-γ antibody [αIFNγ]) or TNF-α (anti-TNF-α antibody [αTNF-α]) or were treated with isotype-matched control MAb. At 72 h postchallenge, the numbers of bacterial CFU in the lung (A) and liver (B) were determined. In parallel, pulmonary levels of NOS2 mRNA were measured by real-time PCR (C). Vaccination significantly decreased the pulmonary and hepatic bacterial burden and increased levels of NOS2 mRNA (∗; $P <$ 0.0001, 0.0001, and 0.01, respectively). In comparison with vaccinated mice treated with isotype control MAb, those treated with anti-IFN-γ displayed significantly increased pulmonary and hepatic bacterial burden and decreased levels of NOS2 mRNA (#; $P <$ 0.02, 0.03, and 0.002, respectively). Vaccinated mice treated with anti-TNF-α also displayed significantly increased pulmonary and hepatic bacterial burden and decreased levels of NOS2 mRNA in comparison with those of vaccinated mice treated with isotype control MAb (*x*; $P <$ 0.0003, 0.0002, and 0.03, respectively). For panels A, B, and C, there were 10 mice per group, and data are pooled from two independent experiments.

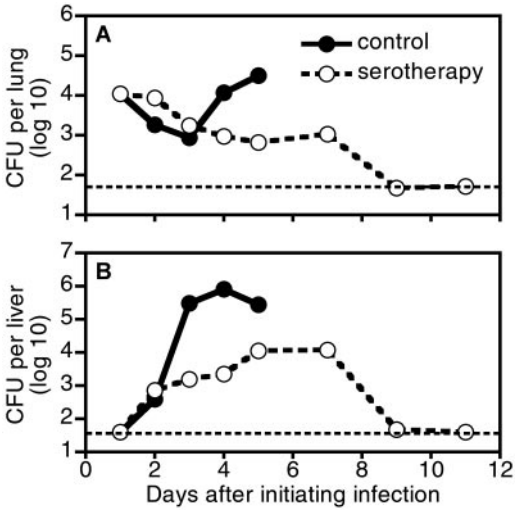


FIG. 3. Passive serotherapy limits bacterial growth. Wild-type C57BL/6 mice were challenged intranasally with *Y. pestis* ($2 \times 10^5$ CFU i.n.). As indicated, mice received convalescent-phase sera (serotherapy) or no additional treatment (control) 18 h later. The numbers of bacterial CFU in the lung (A) and liver (B) were determined at the indicated times postchallenge. Data are means (five mice per group). The broken line depicts the level of detection.

ilar to that of the livers (data not shown). These data suggest that IFN-γ and TNF-α protect against lethal pulmonary infection in actively vaccinated mice by reducing both the pulmonary and extrapulmonary bacterial burden.

In other bacterial infection models, IFN-γ and TNF-α mediate protection, in part, via their capacities to upregulate NOS2 expression (24). To investigate the role of NOS2 in our active vaccination protocol, we measured levels of pulmonary NOS2 mRNA at day 3 postchallenge. We found that vaccinated mice, compared with sham-vaccinated controls, exhibited significantly increased levels of NOS2 mRNA (Fig. 2C, $P =$ 0.006). Depletion of either IFN-γ or TNF-α at the time of challenge significantly suppressed the vaccination-induced upregulation of NOS2 mRNA ($P = 0.001$ and $P = 0.02$, respectively). These results suggest that upregulation of NOS2 expression is one mechanism by which cell-mediated immunity combats lethal pulmonary *Y. pestis* challenge.

**IFN-γ, TNF-α, and NOS2 perform critical protective functions during passive humoral defense against lethal pulmonary *Y. pestis* challenge.** Having defined an important role for classical mediators of cellular immunity during protection conferred by active vaccination, we next investigated whether these mediators also participate in protection conferred by passive serotherapy. The passive transfer of convalescent-phase sera protects naïve wild-type mice against lethal pulmonary *Y. pestis* infection, even when administered 18 h after challenge (29). As shown in Fig. 3A, both serotherapy-treated and untreated mice exhibit similar and gradually decreasing numbers of recoverable pulmonary CFU up until day 3 postchallenge. Pulmonary CFU then continue to gradually decline in the serotherapy-treated mice and fall below the detection limit of our assay on day 9 postchallenge. By contrast, the number of recoverable pulmonary CFU steadily increases in the untreated mice, which become moribund by day 5 postchallenge. We observed similar patterns of growth in the liver, except that differences between treated and untreated mice became evident by day 3 postchallenge (Fig. 3B). We conclude that passive serotherapy promotes a gradual reduction in the bacterial burden in both the lung and extrapulmonary tissues.

To investigate the roles of key mediators of cellular immunity during protection conferred by passive serotherapy, we administered convalescent-phase sera to either gene-targeted IFN-γR-deficient or TNF-α-deficient mice. In comparison with wild-type mice, both IFN-γR-deficient mice and TNF-α-deficient mice exhibited significantly reduced survival (Fig. 4; $P <$ 0.0001). We conclude that the type 1 cytokines IFN-γ and

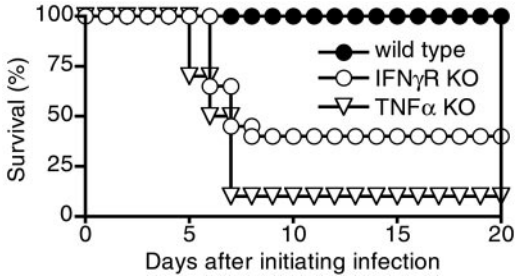


FIG. 4. IFN-γ and TNF-α perform critical protective functions during humoral defense against lethal pulmonary *Y. pestis* challenge. Wild-type and gene-targeted IFN-γR-deficient or TNF-α-deficient mice were challenged intranasally with *Y. pestis* ($2 \times 10^5$ CFU) and treated with convalescent-phase sera 18 h later. In comparison with wild-type control mice, both IFN-γR-deficient mice and TNF-α-deficient mice exhibited significantly reduced survival ($P < 0.0001$ for IFN-γR- and TNF-α-deficient mice; 20 mice each for wild-type and IFN-γR-deficient groups and 10 mice for TNF-α-deficient group). Data are pooled from two independent experiments. KO, knockout.

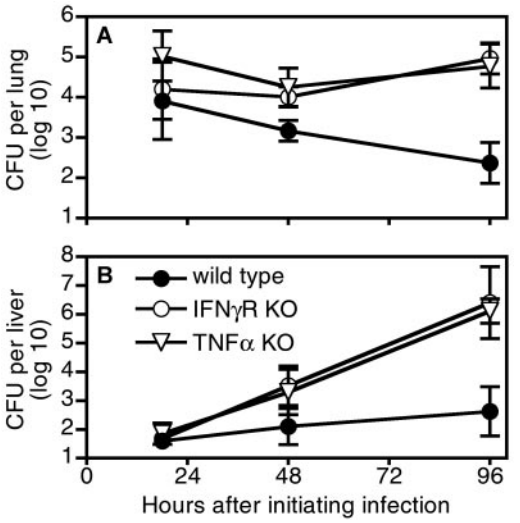


FIG. 5. IFN-γ and TNF-α control bacterial growth during humoral defense against lethal pulmonary *Y. pestis* challenge. Wild-type and gene-targeted IFN-γR-deficient or TNF-α-deficient mice were challenged intranasally with *Y. pestis* ($2 \times 10^5$ CFU) and treated with convalescent-phase sera 18 h later. At the times indicated postchallenge, the numbers of bacterial CFU in the lung (A) and the liver (B) were determined. In comparison with wild-type control mice, IFN-γR-deficient mice exhibited significantly increased numbers of pulmonary CFU at both 48 and 96 h postchallenge ($P = 0.01$ and $P < 0.0001$, respectively). In comparison with wild-type control mice, TNF-α-deficient mice exhibited significantly increased numbers of pulmonary CFU at 18, 48, and 96 h postchallenge ($P = 0.01$, $P = 0.0003$, and $P < 0.0001$, respectively). In comparison with wild-type control mice, both IFN-γR-deficient and TNF-α-deficient mice exhibited significantly increased numbers of hepatic CFU at both 48 and 96 h postchallenge (IFN-γR-deficient, $P = 0.01$ and $P < 0.001$, respectively; TNF-α-deficient, $P = 0.03$ and $P < 0.0001$, respectively). Data are pooled from two independent experiments (10 mice per group). KO, knockout.

TNF-α both contribute to protection conferred by passive serotherapy during pulmonary *Y. pestis* challenge.

To investigate how IFN-γ and TNF-α contribute to protection conferred by passive serotherapy, we determined the numbers of bacterial CFU in challenged mice. Specifically, we determined the numbers of CFU in lungs, livers, and spleens at 18, 48, and 96 h postchallenge. At 18 h postchallenge, the time of serotherapy administration, TNF-α-deficient mice already displayed a modest increase in pulmonary bacterial burden compared with wild-type mice (Fig. 5A; $P = 0.01$). At 48 h postchallenge, both IFN-γR deficiency and TNF-α deficiency significantly increased the bacterial burden in both the lung and liver (Fig. 5; $P < 0.05$ for both organs). These increases were even more pronounced at 96 h postchallenge (Fig. 5; $P < 0.001$ for both organs). Bacterial growth in the spleen resembled that shown for the liver (not shown). We conclude that abrogating either IFN-γ or TNF-α signaling suppresses the capacity of passive serotherapy to limit bacterial growth in both pulmonary and extrapulmonary tissues.

Having demonstrated important roles for IFN-γ and TNF-α during protection conferred by passive serotherapy, we next investigated the role of NOS2. As shown in Fig. 6, we found that passive serotherapy provided NOS2-deficient mice with significantly less protection than wild-type mice (Fig. 6; $P < 0.0001$). We conclude that IFN-γ, TNF-α, and NOS2, key mediators of cellular immunity, all play critical roles during humoral defense against pulmonary *Y. pestis* challenge.

## DISCUSSION

We previously demonstrated that vaccinating B-cell-deficient μMT mice with live *Y. pestis* primes T cells that protect against lethal pulmonary challenge (29). Here, we demonstrated that this cell-mediated protection requires two type 1 cytokines, IFN-γ and TNF-α. In the absence of either cytokine, actively vaccinated μMT mice exhibit significantly increased bacterial burden and acutely succumb to challenge infection. Prior studies have documented the roles of IFN-γ and TNF-α in the clearance of facultative intracellular pathogens, including other yersinia species (2, 3). With regard to *Y. pestis*, Nakajima and Brubaker first documented the protective roles of IFN-γ and TNF-α by demonstrating that their parenteral administration confers naïve mice with significant protection against intravenous *Y. pestis* challenge (28). Subsequently, Elvin and Williamson reported that STAT4-deficient mice vaccinated with F1 and V produce high-titer antibodies but,

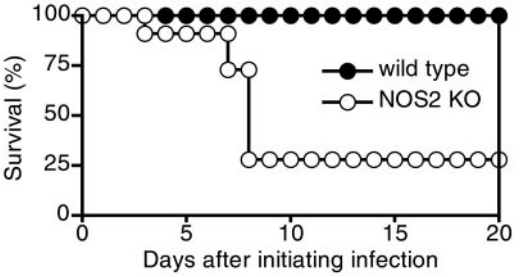


FIG. 6. NOS2 performs a critical protective function during humoral defense against lethal pulmonary *Y. pestis* challenge. Wild-type and gene-targeted NOS2-deficient mice were challenged intranasally with *Y. pestis* ($2 \times 10^5$ CFU) and treated with convalescent-phase sera 18 h. In comparison with wild-type control mice, NOS2-deficient mice exhibited significantly reduced survival ($P < 0.0001$; 10 mice per group). Data are pooled from two independent experiments. KO, knockout.

nevertheless, succumb to subcutaneous *Y. pestis* challenge (8). That finding suggested that type 1 cytokines may contribute to vaccine-mediated protection because STAT4 deficiency is known to impair the generation of type 1 responses (18, 38). In this report, we decisively demonstrated the critical roles of specific key elements of type 1 cellular immunity during vaccine-mediated defense against pulmonary *Y. pestis* challenge.

IFN-γ contributes to antimicrobial defense via a number of distinct mechanisms (35), including the upregulation of NOS2 expression by macrophages, a process that results in the production of nitric oxide and subsequent killing of intracellular organisms (24). In the presence of IFN-γ, TNF-α further upregulates macrophage NOS2 expression (24). We observed increased NOS2 expression in actively vaccinated μMT mice and found that depletion of either IFN-γ or TNF-α suppressed this upregulation. Notably, Pujol and colleagues recently demonstrated that *Y. pestis* organisms possess mechanisms that suppress their intracellular killing by nitric oxide (33). Together, these observations suggest that nitric oxide is detrimental to *Y. pestis* and that upregulation of NOS2 expression is one mechanism by which the type 1 cytokines contribute to cell-mediated defense against pulmonary *Y. pestis* challenge.

Researchers developing plague subunit vaccines have primarily aimed at eliciting protective humoral responses, in part because the passive transfer of specific antibodies suffices to protect mice against lethal pulmonary *Y. pestis* challenge (10, 25). We too found that passive serotherapy suffices to protect against pulmonary challenge. However, we subsequently observed that passive serotherapy largely fails to protect IFN-γR-, TNF-α-, and NOS2-deficient mice. These findings reveal a previously unappreciated dependency of humoral immunity on cellular immune mechanisms during defense against pulmonary *Y. pestis* challenge. Notably, a similar dependency has also been documented for another facultative intracellular pathogen, *Cryptococcus neoformans*, where IFN-γ and NOS2 perform critical protective functions during antibody-mediated defense (5).

How might cellular immunity contribute to humoral defense against pulmonary *Y. pestis* challenge? While pathogenic roles for intracellular *Y. pestis* organisms have yet to be demonstrated during plague, viable *Y. pestis* organisms are found within macrophages in vivo (6, 22) and *Y. pestis* can survive and replicate within macrophages in vitro (15, 33, 37). As such, classical cell-mediated immune mechanisms may aid humoral defense by eradicating intracellular *Y. pestis* reservoirs. At the same time, classical humoral immune mechanisms could directly combat extracellular *Y. pestis* organisms and simultaneously aid cell-mediated immunity both by neutralizing *Y. pestis* virulence factors that dampen cellular responses (4, 28) and by delivering antibody/antigen complexes to B cells, macrophages, and/or dendritic cells, thereby promoting T-cell activation (5, 13, 27, 40). While cell-mediated clearance of intracellular bacteria may be key to humoral defense against pulmonary *Y. pestis* infection, our observations do not, in and of themselves, establish pathogenic roles for intracellular bacteria. Indeed, extracellular organisms dominate pulmonary *Y. pestis* infection (21), and a recent study found that humoral defense against *Bordetella bronchioseptica*, an extracellular bacterium, also requires IFN-γ (31). Thus, further studies will be required to determine whether cellular immunity aids humoral defense by eradicating intracellular *Y. pestis* reservoirs and/or by helping to clear extracellular organisms. To our knowledge, specific mechanisms by which cellular immunity aids humoral defense against extracellular bacteria have yet to be established in vivo. However, one likely possibility is that type 1 cytokines activate and/or recruit phagocytes, thereby enhancing their capacity to capture and destroy opsonized bacteria (7).

In conclusion, we have shown herein that IFN-γ, TNF-α, and NOS2, factors classically associated with cell-mediated defense against intracellular pathogens, play important protective roles during humoral defense against lethal pulmonary *Y. pestis* challenge. These observations strongly suggest that pneumonic plague vaccines should be designed to maximally prime both cellular and humoral immunity.

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